J. Anat. (1989), **162**, pp. 249–261
With 10 *figures*
Printed in Great Britain

Effect of raised serum prolactin on breast development*

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(*Accepted* 2 *June* 1988)

INTRODUCTION

It is well established that serum prolactin plays a major role in breast development. Factors which lead to the elevation of serum prolactin such as pregnancy (Amenomori, Chen & Meites, 1970), administration of dopamine antagonists (Sulman, 1970) and grafting of multiple pituitary isografts (Everett, 1954) also lead to breast growth.

Early qualitative studies (Cole & Hopkins, 1962; Ben-David & Chrambach, 1970) suggested that growth was due to an increase in both the number and size of differentiated lobular structures in the breast, this being accompanied by increased ductular and lobular luminal volume.

Previous attempts to quantify these changes in morphology, however, have suffered from a number of drawbacks. First, and without exception, only a few of the component tissues of the breast have been studied in a single investigation and a distinction between myoepithelium and epithelium has not been made on separate structures (Ben-David, Dirkstein & Sulman, 1965; Ben-David, 1968; Sulman, 1970). Secondly, in almost all cases, unreliable sampling methods have been employed without reference to stereological principles, often employing only single breast pads for study in the rat and mouse (Cowie & Fowley, 1947; Danon, Weller & Sulman, 1970; Sulman, 1970; Silver, 1983), and therefore without determination of changes in the total volumes of components. Finally, no attempt has been made to relate changes in morphology to the level of prolactin stimulus.

The purpose of this study was to provide a reliable, quantitative assessment of the individual components of the breast during breast growth in the rat and to correlate these changes with the level of circulating prolactin.

In view of the studies which have underlined the role of prolactin in mammary tumorigenesis (Lacassagne & Duplan, 1959; Welsch, Jenkins & Meites, 1970), it was hoped that the results obtained would provide an accurate baseline for further investigations now being undertaken to elucidate the mechanisms regulating breast growth.

MATERIALS AND METHODS

Female Sprague–Dawley rats aged 7 weeks and weighing 182 ± 7 g ($\bar{x} \pm$ S.E.) were obtained from A. Tuck & Son, Essex, England, and housed 5 to a cage in a thermostatically controlled room (21 ± 2 °C, temperature ± accepted range) with a constant 12 hour light/12 hour dark cycle (light 07.00–19.00). Animals were fed on a

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standard laboratory diet (Pilbury's rat and mouse breeding diet) and given free access to drinking water. Vaginal smears were taken daily between 08.00 and 12.00 hours and only rats undergoing 3 or more regular 4 day oestrous cycles were chosen for this study. The stage in the cycle was assessed by light microscopic examination of vaginal smears after Papanicoulou staining. The oestrous cycle was defined as: oestrus, the day of cornified cells only; metoestrus, the first day of leukocytes; dioestrus, the second day of leukocytes together with mucus production; pro-oestrus, the day with nucleated cells present in the absence of leukocytes.

Fifty one rats were used for serum prolactin studies. Thirty rats were given perphenazine orally as a 0·01% solution, made up from Fentazin syrup (2 mg perphenazine/ml syrup, Allen & Hanbury Ltd, Greenford, England) in light-tight containers, the solution being renewed every two days. This was commenced 48 hours after a dioestrus smear. No further smears were taken before blood sampling because of the known effects of handling on serum prolactin levels. Twenty one animals were not given perphenazine; sixteen were killed at the beginning of the experiment (four in each stage of the oestrous cycle), and a further five were killed at the end of the experiment. The perphenazine-treated animals were killed in groups of five at 2, 4, 7, 14, 27 and 54 days after the start of the experiment.

All animals were killed rapidly between 13.00 and 13.10 hours by decapitation in a separate room in order to avoid the known effects of stress on the pituitary secretion of prolactin (Krulich, Hefco, Ilner & Read, 1974). Trunk blood was collected from the neck, and serum separated for subsequent prolactin assay. Vaginal smears were also taken from all rats after killing and the stage of the oestrous cycle confirmed for the untreated rats and determined for the perphenazine-treated animals.

Since decapitation can lead to breast tissue loss as well as damage, a separate group of forty female rats was used for morphometric studies. Thirty were given perphenazine 48 hours after a dioestrus smear and killed in groups of five on Days 2, 4, 7, 14, 27 and 54 after the start of treatment as described for the prolactin studies. The remaining ten rats were not given perphenazine and were killed five on Day 0 and five on Day 54 of the experiment. Groups were killed by ether overdose and the skin and breast tissue removed whole and pinned out flat onto cork boards. The tissue was then immersed in chilled methacarn fixative (a mixture of methanol, chloroform and acetic acid at 6:3:1) and fixed for 24 hours at 4 °C. After fixation all breast tissue including the surrounding fat matrix was removed from the skin by blunt dissection. The total volume of breast tissue plus surrounding matrix of each rat was assessed by measuring the volume of methacarn displaced in a volumetric cylinder after tissue immersion.

Before processing, tissue architecture was physically randomised as described previously (Stringer, Wynford-Thomas & Williams, 1982). The tissue was diced into approximately 400 pieces of equal size. The diced tissue was then processed in chloroform and embedded into 5 wax blocks per rat. Each block contained approximately 80 pieces of breast tissue in random orientation within the block. Two serial sections of thickness 4 μm were cut from each block. In one section the myoepithelium was localised by immunocytochemistry, using antimyosin antiserum and an indirect immunoperoxidase technique and counterstained with Harris' haematoxylin (Fig. 1). The remaining sections from each block were stained with Harris' haematoxylin only.

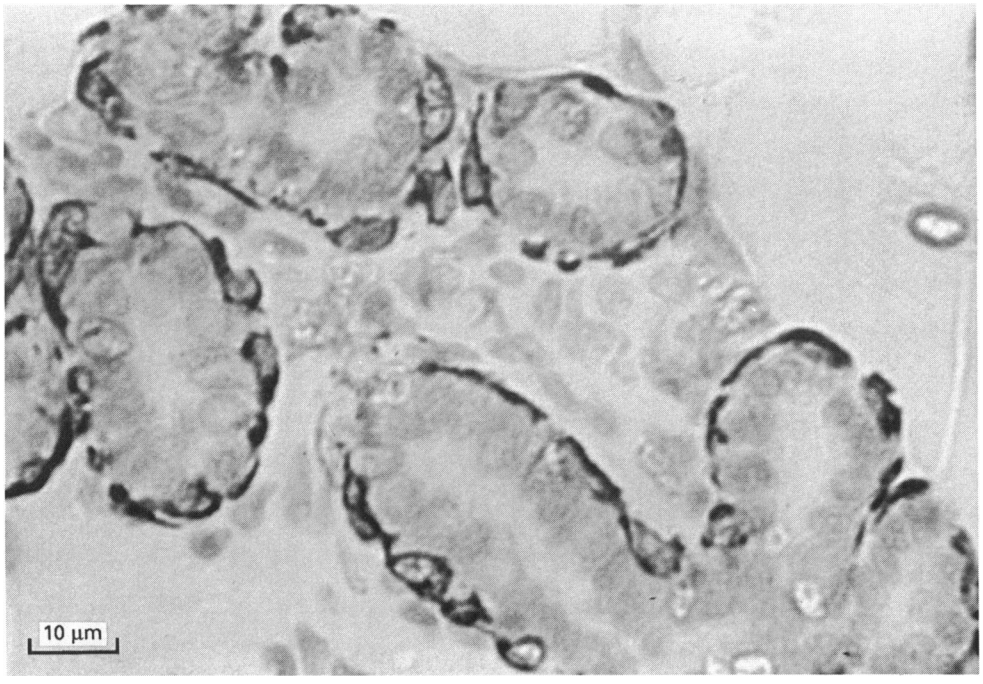

Fig. 1. 4 µm section through normal rat breast. The myoepithelial cells have been localised immunocytochemically using anti-myosin antisera and an indirect immunoperoxidase technique. The section has been counterstained with Harris' haematoxylin.

Microscopic analysis

Components quantified

The epithelial elements of the breast penetrate the subcutaneous fat and, in the rodent during pregnancy, they spread widely through the subcutis virtually joining across the back of the animals. Breast tissue for the purpose of this morphometric analysis was defined as the epithelial component from nipple to alveolus together with the myoepithelial cells, lumen and associated specialised stroma. The relative volume of breast tissue and fat was measured at low magnification and the absolute volume then calculated by reference to the earlier displacement measurement. Four components of breast tissue were then measured at high magnification, as follows.

(1) *Lumen component*: the space surrounded by either alveolar or duct epithelium.

(2) *Epithelial component*: epithelial cells characterised by their morphological appearance, their position in relation to the breast duct and alveolar lumen and their staining properties.

(3) *Myoepithelial component*: defined both by the relationship of the cell to the epithelium and by the immunocytochemical demonstration of intracellular myosin.

(4) *Stromal component*: defined as the connective tissue and blood vessels (including lumen) of the specialised stroma surrounding the epithelium; excluding the surrounding fat cells.

Each component was subdivided into that associated with ducts or ductules and that associated with alveoli (including alveolar buds).

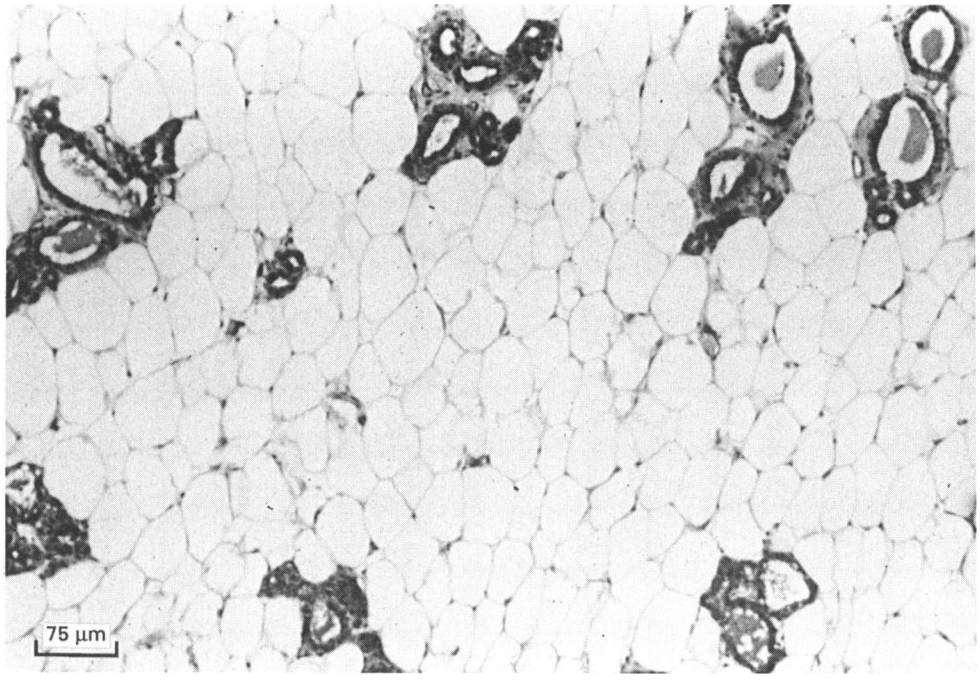

Fig. 2. Low power view of H and E stained 4 μm section through normal rat breast. The majority of the breast pad is fat matrix penetrated by occasional breast epithelial elements. In the unstimulated gland, no marked lumen distension is seen.

Quantitative microscopic analysis

Total breast tissue was quantified semi-automatically with the use of an IBAS II Image Analyser. To do this, sections stained with haematoxylin were viewed at low power (field size of 510×510 μm), the field of view being processed via a video camera into the computer of the image analyser and displayed on the video screen. Breast tissue was discriminated from the fat matrix by the use of a programme designed to distinguish differences in 'grey-level' within the picture. Image analysis discrimination was visually confirmed on the video screen by a colour overlay. Any discrepancies between observed and expected discrimination were edited manually with a hand-held cursor. Once editing was completed, the area of the breast tissue was automatically computed as a percentage of the whole field viewed.

The first whole field from the top left hand corner of each of 50 tissue piece profiles chosen at random was quantified for each rat and the mean percentage area of breast tissue ascertained.

The individual compartments of the breast were quantified manually. Sections were viewed at high magnification ($\times 860$) on a projecting microscope (Reichert Visopan) with the screen overlayed with a square lattice consisting of 100 individual points. Ten fields were viewed from each of 5 sections per rat. Each field (116×116 μm) was selected randomly from a separate tissue piece within the section. Each point within the lattice was scored as either epithelial, myoepithelial, luminal or stromal and was subclassified as either alveolar or ductular. Occasionally points were found lying on the boundaries between compartments. On these occasions, the component to the left and/or above the points was scored. The points scored totalled 5000 for each animal.

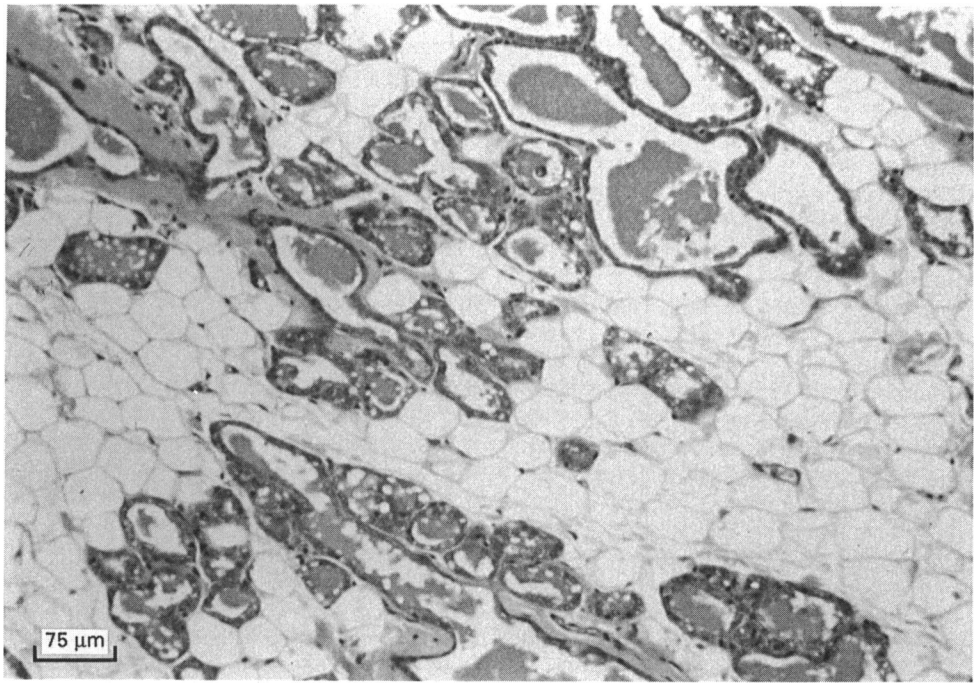

Fig. 3. Low power view of H and E stained 4 μm section through 14 day prolactin-stimulated rat breast. A substantial increase in the breast tissue is seen with marked lumen distension and the presence of fully differentiated lobular structures.

Assay for serum prolactin

Serum samples from each rat were assayed for prolactin by radioimmunoassay using the NIADDK kit. 200 μl serum samples were assayed in triplicate from each rat. Results were expressed in terms of the NIADDK-rPRL-RP-3 standard. Assay precision and sensitivity were ascertained using the method of Ekins (1976): prolactin assay sensitivity in our hands was shown for triplicate samples to be 5·6 ng/ml which was less than 11 % of the overall mean serum prolactin levels present in our untreated control rats. The intra-assay coefficient of variation was found to be 8·2–8·9 % over the 'normal' range and 11·9–13·4 % over the range covering the perphenazine-induced hyperprolactinaemic rat serum concentrations.

Statistical treatment of results

Results are expressed as the mean $\pm$ standard error unless otherwise stated. Where tests of significance were made Student's *t*-independent test was used.

RESULTS

The vaginal smears taken from groups of control rats on Days 1–4 at the start of the experiment showed the expected positions in the oestrous cycle predicted from the vaginal smears taken two days prior to killing. Four groups of four rats were in each of the four stages of the oestrous cycle.

The effects of perphenazine administration on vaginal smear patterns of rats during the first 7 days of treatment were variable both within and between groups. Ten of the vaginal smears taken showed patterns similar to dioestrus, three showed a metoestrus

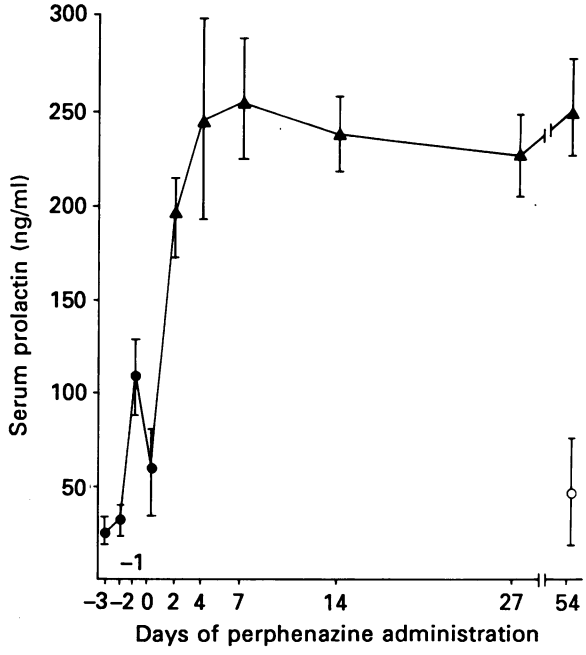

Fig. 4. Effect of oral administration of perphenazine on rat serum prolactin ($\bar{x} \pm$ S.E.M. 4 or 5 rats/point; see text). ●—●, normal rat serum prolactin levels at 13.00 hours on day of metoestrus (Day 3), dioestrus (Day 2), pro-oestrus (Day 1) and oestrus (Day 0). ▲—▲, serum prolactin levels at 13.00 hours in rats administered perphenazine orally for varying times up to 54 days. ○, normal rat (unselected for phase of oestrus) serum prolactin levels maintained as controls for the 54 day perphenazine-treated group.

pattern and for the remaining two rats, a pattern equivalent to pro-oestrus was seen. In general, as the treatment time increased, the tendency for the vaginal smears to give a predominantly dioestrus appearance also increased. None of the treated rats showed a vaginal smear pattern equivalent to the oestrus stage at any time point.

The effects of the oestrous cycle and oral administration of perphenazine on rat serum prolactin are illustrated in Figure 4. The serum prolactin levels of untreated rats sampled at 13.00 hours on each day of the 4 day oestrous cycle were: $24{\cdot}3 \pm 6{\cdot}2$, $29{\cdot}8 \pm 7{\cdot}4$, $108{\cdot}6 \pm 24{\cdot}4$, and $56{\cdot}3 \pm 18{\cdot}4$ for metoestrus, dioestrus, pro-oestrus and oestrus respectively.

The pro-oestrus prolactin levels were significantly greater than those of the metoestrus ($P < 0{\cdot}02$) and dioestrus ($P < 0{\cdot}05$) phases. No significant difference was seen between prolactin levels of pro-oestrus and oestrus, nor between oestrus and metoestrus or dioestrus.

Oral administration of perphenazine (0·01%) led to a marked rise in serum prolactin. This was seen at the first time point sampled (2 days) when it was already significantly increased when compared to the level of prolactin at any phase of oestrous cycle (metoestrus $P < 0{\cdot}001$, dioestrus $P < 0{\cdot}001$, pro-oestrus $P < 0{\cdot}05$, oestrus $P < 0{\cdot}01$). Further rises occurred at 4 and at 7 days after commencing perphenazine administration, and a sustained high level was maintained until the study was concluded at 54 days. The prolactin levels of the control group of animals killed at the end of the experiment were not significantly different from the levels of the control animals killed at the start of the experiment.

The effect of orally administered perphenazine on rat body weight is shown in

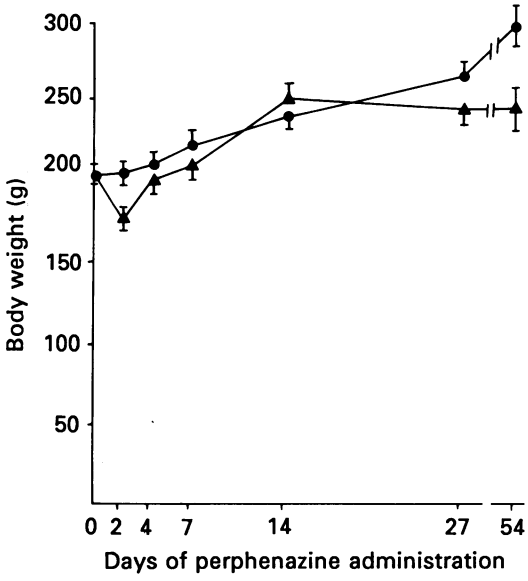

Fig. 5. Effect of oral administration of perphenazine (0·01 % in drinking water) on rat body weight ($\bar{x} \pm$ S.E.M. 5 rats/point). ●—●, untreated control rats; ▲—▲, rats given oral perphenazine.

Figure 5. Body weights of the treated groups were compared with age-matched untreated control rats for each time-point. A significant ($P < 0{\cdot}05$) fall in the body weights of treated rats was seen after two days of perphenazine administration, but this returned to basal values by Day 4 of the treatment. From Day 4 of treatment, the weight gains of both the control and treated groups were similar with no significant difference between the groups at matched time-points up to 27 days. The mean body weight of the 54 days perphenazine-treated group, however, was slightly but significantly ($P < 0{\cdot}05$) lower than that of the age-matched untreated control group.

The effect of raised serum prolactin on total rat breast volume is shown in Figure 6. A significant ($P < 0{\cdot}01$) 3·4-fold increase in breast volume was seen at 4 days after beginning treatment, with a continued rise up to an 8·9-fold peak by Day 14. A slight but significant ($P < 0{\cdot}05$) fall in the 14 days value was seen at 27 days but no significant difference was seen between the 14 days and 54 days levels, nor between the 27 days and 54 days levels.

Changes in the volumes of the epithelial components are shown in Figure 7. A small but significant ($P < 0{\cdot}05$) 2·6-fold increase in the volume of the ductular epithelium was found 4 days after the start of perphenazine administration. This remained elevated for the duration of the experiment but no further significant increases were seen. A highly significant ($P < 0{\cdot}001$) 5·6-fold increase in the alveolar epithelium volume was seen by Day 4 with a continued rise to a 15·6-fold peak by Day 14. A slight but significant ($P < 0{\cdot}05$) fall from this peak occurred by Day 27 but as with the total breast volume, this was later reversed so that the 54 day measurement was not significantly different from either the 14 or 27 day levels.

Myoepithelium component volume changes in response to serum prolactin elevation are shown in Figure 8. A significant ($P < 0{\cdot}05$) 2·1-fold rise in the ductular myoepithelial component was also seen by Day 4 and it continued to rise to reach a 2·9-fold ($P < 0{\cdot}01$) peak by Day 7. The peak was not sustained, however, and the

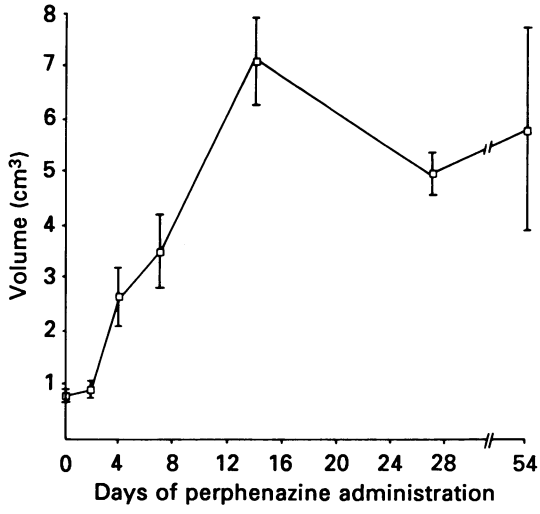

Fig. 6. Effect of perphenazine administration on total rat breast volume ($\bar{x} \pm$ S.E.M. 5 rats/point).

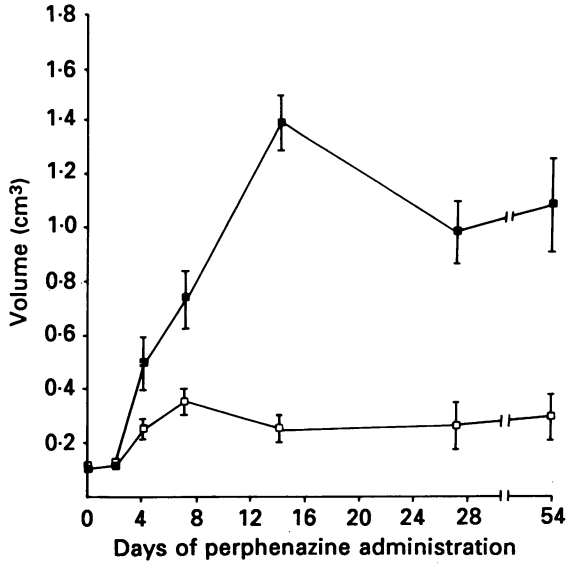

Fig. 7. Effect of perphenazine administration on breast alveolar (■—■) and ductular (□—□) epithelial volume ($\bar{x} \pm$ S.E.M. 5 rats/point).

ductular myoepithelial volume declined to just above normal levels at the 14, 27 and 54 day time-points. A significant ($P < 0{\cdot}05$) 3·7-fold increase in the alveolar myoepithelial compartment volume was seen 4 days after beginning treatment. This rose further to a 7·2-fold ($P < 0{\cdot}001$) peak by Day 14 and remained at this level throughout the rest of the treatment period.

Both the ductular and alveolar lumen compartment volumes rose in response to raised serum prolactin (Fig. 9). A marked 63-fold increase ($P < 0{\cdot}001$) in the alveolar lumen compartment volume was seen by Day 14 but this declined to a 36-fold increase by Day 54. A significant but more gradual increase in the lumen compartment was also seen in the ducts leading a stable 16-fold ($P < 0{\cdot}001$) plateau by Day 14.

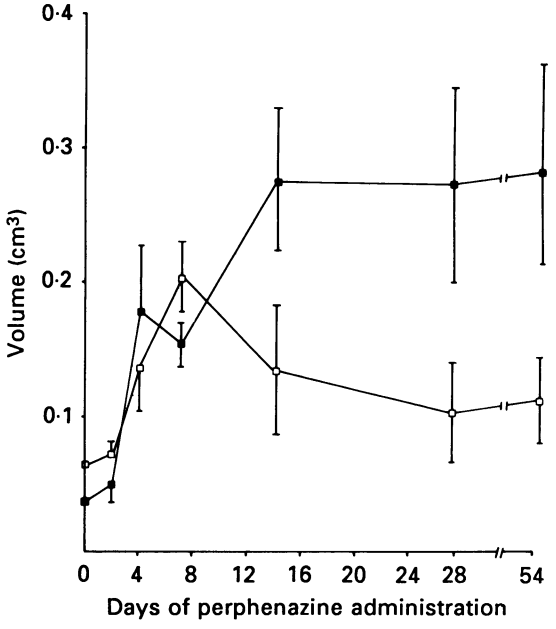

Fig. 8. Effect of perphenazine administration on breast alveolar (■—■) and ductular (□—□) myoepithelial volume ($\bar{x} \pm$ S.E.M. 5 rats/point).

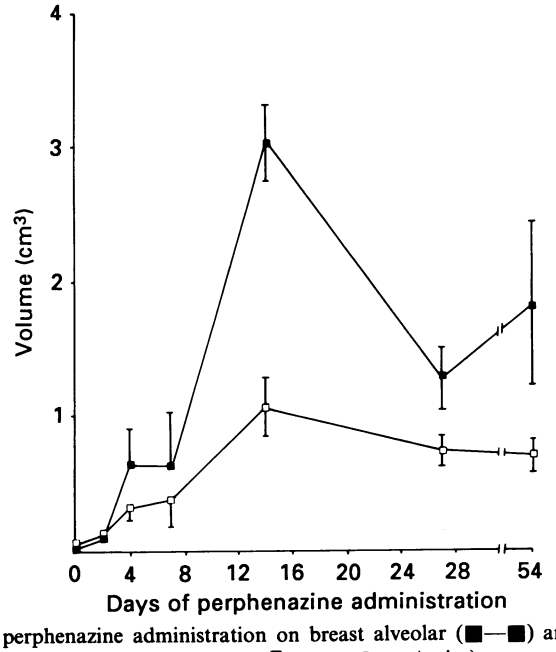

Fig. 9. Effect of perphenazine administration on breast alveolar (■—■) and ductular (□—□) luminal volume ($\bar{x} \pm$ S.E.M. 5 rats/point).

The stromal volume related to alveoli rose to a 3·7-fold ($P < 0{\cdot}001$) plateau by Day 14 of treatment (Fig. 10). A significant ($P < 0{\cdot}01$) 2·1-fold increase in the duct stromal volume was also seen by Day 7, with no further significant changes by Day 54.

No significant changes in any breast components were seen between Day 0 and Day 54 control groups.

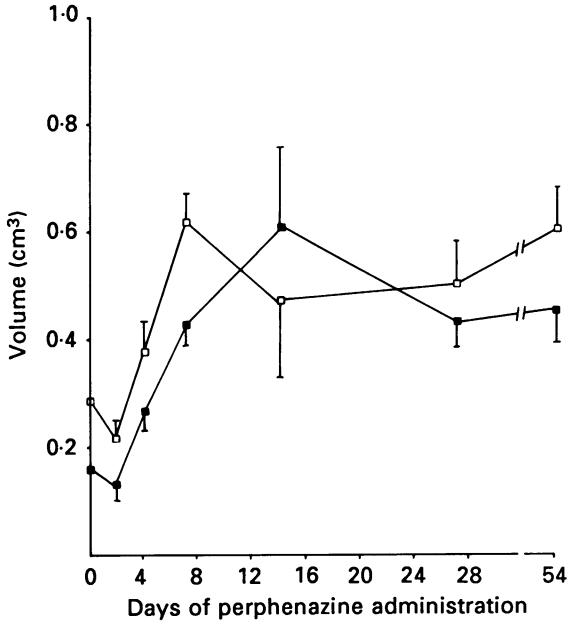

Fig. 10. Effect of perphenazine administration on breast alveolar (■—■) and ductular (□—□) stromal volume ($\bar{x} \pm$ S.E.M. 5 rats/point).

DISCUSSION

Our study showed serum prolactin levels in normal rats to vary at different times of the oestrous cycle. Investigations performed by other workers have conclusively demonstrated an oestrous rhythm of prolactin in both 4 and 5 days cycling rats (Gay, Midgley & Niswender, 1970; Midgley, Niswender, Gay & Reichert, 1971; Butcher, Collins & Fugo, 1974). Also, a circadian rhythm of prolactin has been reported which differs at different times of the cycle (Butcher, Fugo & Collins, 1970). We have not investigated the oestrous or circadian rhythms but as in other studies, high serum prolactin levels were found during pro-oestrus, our reported levels being comparable to those of other workers.

Oral administration of perphenazine led to a dramatic increase in serum prolactin. A highly significant ($P < 0{\cdot}001$) 8·8-fold elevation above basal values was seen at the first time-point sampled and was maintained for the duration of the experiment. Prolactin elevation caused a disruption of the oestrous cycle, leading to a predominantly dioestrus appearance of vaginal smear patterns after the first few days. The dioestrus pattern induced by elevated prolactin has been reported previously (Ben-David, 1968) and is most likely due to the loss of the gonadotrophic surge which is thought to be negated by hyperprolactinaemia (Muralidhar, Maneckjee & Moudgal, 1977). Figure 5 shows the effect of orally administered perphenazine on rat body weight. A small but significant fall was seen after 2 days and 54 days of treatment but otherwise the dopamine antagonist was well tolerated.

Hyperprolactinaemia led to considerable changes in breast morphology during the first few days of serum prolactin elevation. Structures common to unstimulated breast tissue, notably the terminal end buds, lateral buds and terminal ducts (Russo, Tay & Russo, 1982) virtually disappeared by Day 7, being replaced by the more differentiated

alveolar buds and lobular structures. By Day 14 non-ductular breast epithelium consisted almost entirely of fully differentiated lobules, each containing large numbers of individual alveoli. A noticeable increase in the lumen volume of alveoli could be seen by Day 14 (Fig. 3). The qualitative microscopic changes in breast tissue in response to elevated serum prolactin were similar to those reported previously (Sulman, 1970).

Although an increase in breast tissue volume was obvious on subjective light microscopy, there was no significant difference in the volumes of breast tissue with its accompanying fat between any of the groups. Studies by Cole & Hopkins (1962) also showed no significant difference in the wet weight of abdomino-inguinal breast pads taken from untreated and prolactin-treated rats, despite very significant increases in DNA content in response to daily prolactin injections. It is clear from these investigations therefore that the total breast fat pad volume/weight cannot be relied upon as an indicator of true breast tissue volume. Quantitative microscopic analysis must therefore be employed to determine changes in breast volume independent of the surrounding fat matrix.

Unlike previous investigations concerned with changes in breast growth and differentiation in response to prolactin stimulation, we have randomly sampled the entire breast tissue complement included within the fat matrix. This, along with the measurement of the tissue sample volume, has enabled us to provide reliable estimates of changes in the total breast volume. Furthermore, we have studied the changes in eight separate components: epithelium, myoepithelium, lumen and stroma each being subdivided into either ductular or alveolar compartments.

The results show a highly significant ($P < 0{\cdot}001$) 9·2-fold increase in total breast volume from $0{\cdot}80 \pm 0{\cdot}21$ cm^3 in the untreated group to $7{\cdot}15 \pm 0{\cdot}78$ cm^3 after 14 days stimulation. Other workers have shown that short term serum prolactin elevation in the rat leads to mammary gland differentiation (Ben-David & Chrambach, 1970; Sulman, 1970) but have not quantified breast volume changes or considered the ducts and alveoli as independent compartments.

Separation of the breast components into different compartments has clearly identified different patterns of responses. Of particular interest is the finding that the epithelial, myoepithelial and stromal responses of the ducts resembled each other but differed from the epithelial, myoepithelial and stromal responses of the alveoli. The ductal responses reached an early relatively low peak: 2·6-fold increase by 4 days for duct epithelium, 2·9-fold increase by 7 days for duct myoepithelium and 3·1-fold increase by 7 days for duct stroma. In contrast, the alveolar responses reached a late peak, and showed a greater increase: 15·6-fold by 14 days for alveolar epithelium, 7·2-fold increase by 14 days for alveolar myoepithelium and an 8·7-fold increase by 14 days for alveolar stroma. This suggests that the separate but coordinated responses seen for these three compartments in duct and alveolar tissue may be regulated by local controlling mechanisms. More detailed analysis however, including cell turnover studies, will be required to show whether not only the tissue volumes but also mitotic rates are coordinated.

The changes in lumen volume are of less interest than are those of the cellular compartments but the alveolar lumen showed the expected increase from a very low to a high level. Identification of the various components proved easy, although the recognition of intracellular myosin by immunocytochemistry proved essential for the quantification of the myoepithelial compartment.

Although there have been studies on the myoepithelial cell during breast differentiation and development (Radnor, 1972*a*, *b*) we believe this to be the first

investigation to quantify changes in total myoepithelium volume in normal and stimulated breast.

SUMMARY

The effect of serum prolactin elevation on the growth and development of the rat breast was investigated. Oral administration of the dopamine antagonist, perphenazine, led to a 5–10-fold elevation of serum prolactin after two days of treatment which was maintained for the 54 days of study. A significant ($P < 0.01$) 3·4-fold increase in total breast volume was seen by Day 4 of serum prolactin elevation. Breast volume continued to rise up to Day 14 reaching an 8·9-fold peak ($P < 0.001$) which was maintained for the duration of the experiment. Epithelial, myoepithelial, lumen and stromal volume changes in the ductular and alveolar compartments were quantified separately. Highly significant ($P < 0.01$) volume increases were seen in all components within the first few days of prolactin elevation.

Similar time courses of the growth response to elevated serum prolactin were seen in the ductal tissues reaching an approximate 3-fold peak by 7 days in duct epithelium, myoepithelium and duct stroma. Time coordinated growth responses were also seen in the alveolar tissues with larger (7–15-fold) increases in alveolar epithelium, alveolar myoepithelium and alveolar stroma, reaching a peak by 14 days.

We would like to thank the Medical Research Council for grant support. We are grateful to Dr Parlow of the NIADDK, NIH, Bethesda, U.S.A., for the supply of the reagents used in this study.

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